Directed protein orientation by site-specific labeling

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***Abstract*—Membrane proteins like bacteriorhodopsin possess native amino acid residues that can be used for site specific labeling towards directed orientation onto solid substrates. Utilizing the lysine 129 residue found in bacteriorhodopsin, biotinylation of the extracellular surface of the purple membrane can be achieved. Furthermore, this method can be applied to other protein transporters to fabricate large-area planar arrays of oriented protein towards the measurement of coupled protein functionality.**

Keywords-bacteriorhodopsin, protein orientation, biotinylation

I. Introduction

Bacteriorhodopsin is the sole protein found in the purple membrane of *Halobacterium Halobium* and is a proton pump that can be actuated by green light when the retinal chromophore undergoes a conformational change from the all-trans to the 13-cis state. Due to the extreme conditions in which the *Halobacterium* resides (>80°C), the bacteriorhodopsin remains functional at very high temperatures and a wide range of pH conditions (1-12). This excellent durability and chemical resistance of the protein makes it an ideal candidate for deposition onto solid substrates in that bacteriorhodopsin can continue to function even in the absence of liquid media. In alkaline conditions, the extracellular lysine 129 (K129) becomes the only amenable residue towards bioconjugation [1]. Also, it has been previously established that these labeled purple membrane constructs can successfully interact with streptavidin-labeled lipid heads [2]. As such, K129 biotinylation using a long-arm biotin crosslinker, coupled with its interaction with a streptavidin-gold coated surface, would then be used to fabricate large-area planar arrays of oriented protein that possess a virtually irreversible bond between the biotin and streptavidin. A coupled protein transporter that is currently being used is an engineered strain of the cytochrome c oxidase from *R. sphaeroides* which possesses a pre-existing engineered 6X-his residue on the extracellular surface that can be conjugated with a nickel sulfate-charged biotin, X-NTA crosslinker to bind with streptavidin. The viability of cytochrome c oxidase is conveyed through the absorbance spectra of the heme *a* group which displays distinctive absorbance peaks at ~ 606 nm. In order to establish protein viability while adsorbed to the solid substrates, UV-vis spectrophotometry was used to detect spectral shifts in chromophore absorbance. Results have indicated that purple membrane can be successfully labeled with biotin while native spectral properties remain unchanged after biotinylation and deposition onto solid substrates.

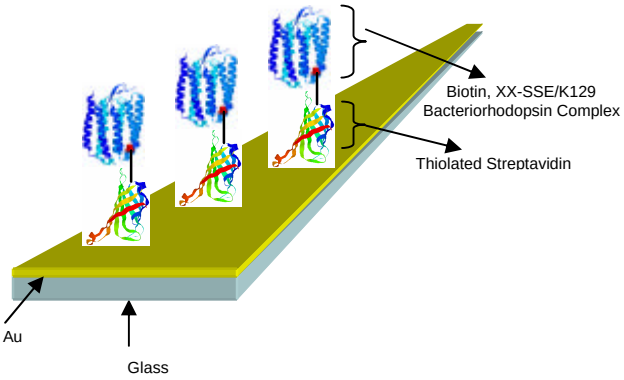

Figure 1. A schematic diagram is shown here depicting the conjugation between a biotinylated K129 residue and thiolated streptavidin.

II. Materials and Methods

Purple membrane from *Halobacterium Halobium* was purchased from Sigma and solubilized to 2mg/ml in 25mM phosphate buffer and 10% Triton X-100. The solution was then vortexed thoroughly, sonicated for 30 seconds, and then incubated at room temperature for 24 hours.

Protein labeling was achieved by using biotin,XX-SSE (Molecular Probes). The 2mg/ml solution (pH 9.5) of purple membrane was stirred at room temperature with 100µl of 10mg/ml biotin,XX-SSE dissolved in dimethylformamide as well as 100µl of $NaPO_4$ for 2 hours. The protein solution was then dialyzed against 2 changes of 0.01M PBS solution, pH 7.2, for 48 hours. For biotinylation of cytochrome c oxidase The extent of biotinylation was tested using the HABA-avidin method [3].

Thiolation of the streptavidin was performed by incubation of a 1mg/ml solution of streptavidin (Sigma) with a 15% solution of 2-iminothiolane (Sigma) and 10X ITL Buffer (0.5M Triethanolamine-HCl, 10mM $MgCl_2$,10mM KCl) at room temperature for 30 minutes. 50nm of gold was evaporated onto glass slides which were subsequently incubated with the thiolated streptavidin solution for 30 minutes. Samples were then washed using Nanopure water and dried under N_2 streams. The substrates were then incubated with the labeled purple membrane for two rounds of 30 minutes each, with each round followed by a Nanopure rinse and N_2 drying.

Analysis of chromophore viability was performed using UV-vis spectrophotometry (Beckman) with a wavelength scan range of 250-700nm.

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III. Results and Discussion

The extent of biotinylation was analyzed using the conventional HABA-avidin method that is a colorimetric assay that can be used to detect absorbance shifts based upon the interaction between biotin and streptavidin in the UV spectrophotometer. Based upon the streptavidin/biotin interaction in the spectrophotometer, the labeling efficiency was calculated to be ~0.17 moles biotin/mole of protein in solution. As the dialysis of unbound biotin was carried out for 48 hours, it could be reasonably assessed that the remaining biotin that interacted with the HABA-avidin was bound to the purple membrane. A sample of Triton X-100 solubilized purple membrane was inserted into a UV-vis spectrophotometer to analyze the spectral peaks present, which yielded absorbance increases at both 278nm and 563nm, which are the characteristic peaks for retinal (Figure 2a). After the biotinylation, it could be seen that the peaks were unshifted and the slight decrease in intensity was simply due to the slight dilution of the sample after dialysis (Figure 2b). It could clearly be seen that the biotinylation did not have any major effect on the excitation of the retinal.

Direct adsorption of streptavidin to gold surfaces is maintained by electrostatic interactions and Van der Waals forces. It was previously established using surface plasmon resonance that thiol-Au interactions are significantly stronger than direct adsorption confirmed by higher resonance units for thiol-Au bond. This is due to the fact that the thiol-gold interaction is believed to be a covalent bond which is stronger than electrostatic adsorption [4]. As mentioned by Chen et. al, the biotin-streptavidin interaction is so strong ($?_{\alpha}=10^{15}$ M^{-1}) that that it is virtually irreversible. After incubation of the labeled purple membrane on the streptavidin, the UV-vis measurements indicated that protein had adsorbed to the substrate surface due to the fact that the spectral peaks indicating retinal shift from the all-trans to 13-cis state were present at both 278nm, and ~563nm. When purple membrane is adsorbed to a surface and not residing in solution, the dehydration of a Schiff base results in a shift in the wavelength (~560nm) where absorbance spikes are found [5]. Figure 3 shows the peaks at both 278nm and ~563nm of adsorbed purple membrane.

In order to achieve coupled protein function between the bacteriorhodopsin and the cytochrome c oxidase, directed orientation of both proteins onto a solid substrate would need to be accomplished. As the cytochrome c oxidase uses extracellular protons in order to facilitate electron transport, orientation is essential for proper usage of the BR-created proton gradient. The engineered strain of cytochrome c oxidase contains an extracellularly engineered 6X-his residue that is amenable to bioconjugation using a nitrilotriacetic acid (NTA) arm that can be charged with nickel sulfate and bound to the 6X-histidine.

We have labeled the 6X-histidine residue with biotin,X-NTA which is a biotin linked to an NTA-conjugated arm that can bind to the extracellular cytochrome oxidase surface. Furthermore, UV-vis was able to detect the viability of the heme *a* chromophore of cytochrome c oxidase while adsorbed to a solid substrate.

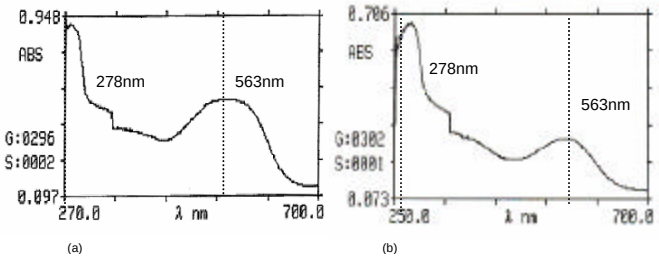

Figure 2. The figure in 2a demonstrates the native PM spectral properties with visible absorbance increases at 278nm and 563nm. The same peaks remain unshifted in the K129/biotin,XX-SSE sample as seen in 2b. Slight dilutions were made to K129 samples during dialysis for removal of unbound biotin.

Figure 4 shows the presence of the spectral peaks of both bacteriorhodopsin (278nm and 563nm), as well as heme *a* spectral peaks at 606nm and 630nm, all adsorbed to the same surface.

Several methods of protein adsorption have been attempted which have exploited various properties of the purple membrane in an effort to orient the purple membrane. We have previously mediated the directional adsorption of purple membrane to solid surfaces using electrostatic orientation, where alternating polycationic/anionic layers of material were sequentially adsorbed onto a surface. Via KOH hydrolysis on a glass surface, the slides were given a slightly negative charge on which a commercial polycation, polydiallyldimethyl ammonium chloride (PDAC) was deposited by incubation for five minutes [5]. At pH 9.4, both the extracellular and cytoplasmic sides of the purple membrane retain a negative charge, but the cytoplasmic side is believed to possess a higher density of negative charges at pH levels above 5 [6]. As such, the cytoplasmic side would then adsorb onto the positively-charged PDAC monolayer. PDAC/PM multilayers could then be sequentially layered in a stacking configuration where gradual increases in absorbance levels at 278nm and 563nm were seen for each additional multilayer adsorbed. The electrostatic orientation method proved to be very easy in practice, and presented the least amount of harsh conditions or treatments to the purple membrane in that no external field was required to orient the protein, as required by the electric field sedimentation technique (EFS), and chemical modification was not necessary. However, for the purposes of our experiments, which call for only a single layer of protein in order to eventually measure proton pumping, a higher binding affinity between the protein and substrate is required in order to fabricate complete monolayers that display increased continuity and increased resistance to washing, and processing conditions, etc. Furthermore, even though adsorption is easily accomplished through electrostatics, orientation efficiency is not guaranteed because both the cytoplasmic and extracellular sides of the purple membrane are negatively charged. Methods like the site-specific labeling display increased efficiency at asymmetric tagging of the protein to enable directional adsorption.

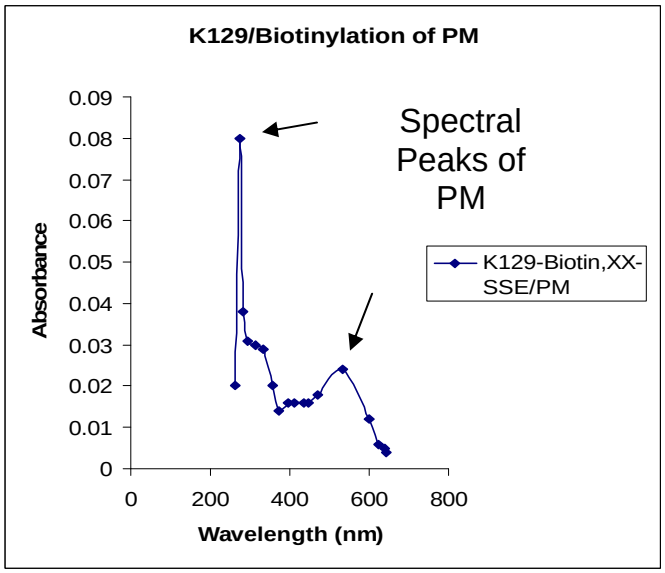

Figure 3. Directed adsorption of labeled purple membrane onto a streptavidin-coated solid support results in preservation of chromophore viability.

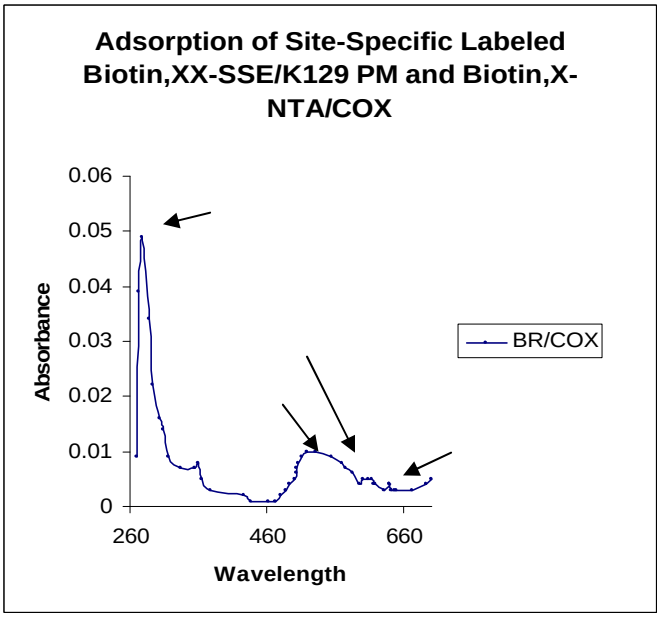

Figure 4. Adsorption of site-specifically labeled purple membrane and cytochrome oxidase is seen by the presence of absorbance peaks at 278nm,563nm(Retinal), and 606nm,637nm (Heme).

The Langmuir-Blodgett deposition method has also been explored with respects to direct adsorption of purple membrane monolayers onto solid supports [7]. However, it has recently been suggested that the orientation at the air-water interface is random which would preclude its applicability towards maximizing proton pumping measurements [8].

In evaluating the various methods available for orienting and adsorbing purple membrane to solid supports, site-specifically labeling the K129 residue with biotin, XX-SSE, or the extracellular 6X-his tag on the cytochrome c oxidase have proven to be easily achievable with concentrations of 0.17mol/mol protein. Furthermore, since the biotin-streptavidin binding affinity is irreversible, opportunities to strongly anchor the protein onto the support are presented.

IV. Conclusions

In this work we have successfully solubilized the purple membrane from *Halobacterium Halobium* and at alkaline conditions, the K129 residue was specifically labeled with a biotin, XX-SSE crosslinker based upon site-specific labeling phenomena established by Henderson et. al. UV-vis measurements were able to show that spectral properties of the protein were unchanged as a result of biotinylation. Also, adsorption onto solid supports resulted in maintenance of chromophore absorbance increases at the characteristic peaks. Subsequent trials included the adsorption of multiple proteins onto one solid support towards the buildup of a couple protein transport system.

Various methods of protein orientation have been attempted in our work that have included electrostatic orientation, as well as Langmuir-Blodgett deposition. While each method has successfully adsorbed protein to a solid support, Chen et. al was able to demonstrate an 85% orientation efficiency using a lipid-based biotin streptavidin configuration.

The thiolated streptavidin that our work has utilized results in covalent bonding to the substrate which we believe further enhances the integrity of the streptavidin monolayer, and consequently, the purple membrane.

Future work will consider the measurement of proton pumping through porous media using the site-specific labeling method mentioned here in order to maximize unidirectional proton flow. Furthermore, coupled protein functionality will also be assessed.

Acknowledgements

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